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The Role of Stromal Cells in the Expression of Interstitial Collagenase (Matrix Metalloproteinase-1) in the Invasion of Gastric Cancer

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Background and Objectives: In one of the steps of tumor invasion and metastasis, tumor cells must invade surrounding tissues and degrade the components of the basement membranes. Interstitial collagenase (matrix metalloproteinase-1: MMP-1) has been also investigated in relation to cancer invasion and metastasis.
Methods: We investigated the formation and mechanism of MMP-1 expression using gastric cancer cell lines and gastric fibroblasts derived from normal gastric mucosa by ELISA and immunohistochemistry.
Results: Production of MMP-1 protein in gastric fibroblasts was stimulated significantly by adding the conditioned medium of MKN-74. Localization of MMP-1 protein in the xenografted gastric cancer cell lines was heterogeneous according to different cell lines.
Conclusions: These results suggested that the production of MMP-1 protein in tumor invasion was regulated by interaction between stromal cells, particularly fibroblasts, and tumor cells.
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KEY WORDS: matrix metalloproteinase-1; collagenase; gastric cancer; stromal cell; fibroblast

INTRODUCTION

Tumor invasion and metastasis are very complex and dynamic processes depending on an interaction between tumor and host tissue cells [1]. In one of the steps of this process, tumor cells must invade surrounding tissues and degrade the components of the extracellular matrix. In 1946, Fischer [2] first described the involvement of proteolytic activity during cancer invasion. To date, more than 14 members of the matrix metalloproteinase (MMP) family have been identified. Collagenase was first described and demonstrated to be the molecule responsible for tail involution in tadpoles [3]. Interstitial collagenase (matrix metalloproteinase-1: MMP-1) has been also investigated in relation to cancer invasion and metastasis [4].

Gastric cancer is known to have various histological and macroscopic types [5], and the pattern of invasion and metastasis varies according to each type. In this study, we investigated the regulatory mechanism of MMP-1 expression using gastric cancer cell lines and gastric fibroblasts.

MATERIALS AND METHODS

Cell Lines

We used three gastric cancer cell lines [6] in this study: MKN-74, a well differentiated adenocarcinoma line; St-40, a well differentiated adenocarcinoma line; and St-4, a

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poorly differentiated adenocarcinoma line. St-40 and St-4 were established at the Pathology Division of the National Cancer Center Research Institute and maintained by serial transplantation into nude mice in our laboratory.

Mice

Male BALB/c-*nu/nu* mice, originated from the Central Institute for Experimental Animals (Kawasaki, Japan), were obtained from CLEA Japan. Inc., Tokyo, Japan. Mice, aged 6 to 8 weeks, were used for the experiment.

Human Gastric Fibroblasts

The human gastric fibroblasts were derived from normal gastric mucosa which was obtained from the surgical specimen of whole stomach from a 48-year-old female with scirrhous gastric cancer on 28 October 1994. The primary culture was as follows: the primary tissues were excised under sterile conditon and placed in Hanks' balanced salt solution (HBSS, GIBCO, Gaithersburg, MD). Tissue specimens were minced with forceps and scissors into small pieces and incubated in sterile centrifuge tube with bacterial collagenase (5 mg/ml in HBSS) for 1 hour at 37°C, followed by adding trypsin (0.25%, 10 ml, GIBCO) and stirring for 30 minutes in cell suspension flask with stirring bar, at room temperature. The sample was filtered through a sterile gauze pad and spun for 5 minutes (1,500 RPM). After the supernatant was discarded by pipette, the cells were washed in HBSS twice by centrifuging at 1,500 RPM and cultivated in 10 cm culture dishes with Dulbecco's modified Eagle's medium (DMEM, GIBCO), 10% fetal calf serum (FCS, GIBCO), 0.5% pyruvate, 1% glutamine, penicillin (100 IU/ml), streptomycin (100 mg/ml), and placed in an incubator at 37°C in an atomosphere of 5% CO_2 in air. After about 2 weeks, with a medium change every 48 hours, fibroblast-like cells grew to confluency in a monolayer, and the serial passages were then carried out every 5–7 days thereafter. The fibroblasts were used for the experiments before the seventh passage of the cells in culture.

Cell Culture

We examined the in vitro interaction of tumor cells and fibroblasts and the expression of MMP-1. MKN-74 cells were grown to 80% confluency in six-well plates with RPMI 1640 containing 10% FCS. After the medium was harvested, MKN-74 cells were washed three times with HBSS, and the medium was replaced with 2 ml of serum-free RPMI 1640 containing 0.2% lactalbumin hydrolysate (LH). After 3 days, the conditioned medium was collected and filtered.

Gastric fibroblasts were grown to 80% confluency in six-well plates with DMEM containing 10% FCS. After the harvest of medium, fibroblasts were washed three times with HBSS, and the medium was replaced with 2 ml of serum-free DMEM containing 0.2% LH and 0.5 ml conditioned medium of MKN-74. After 1, 3, 5, and 7 days, these conditioned media of fibroblasts were collected and filtered. The samples were stored at −20°C until the protein (MMP-1) determination.

We counted the number of fibroblasts at each collection day.

Tissue

Subcutaneous tumor tissues (St-4, St-40) in nude mice were resected aseptically. Specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 1 hour at room temperature and embedded in paraffin. Sections were cut at 5 μm, mounted on microscopic slides, dried thoroughly for 3 hours on a slide warmer at 42°C, and stored at 4°C.

Immunohistochemistry

The primary antibody to MMP-1 was a murine monoclonal antibody, which was kindly provided by Dr. Yasunori Okada, Kanazawa University. Secondary antibody was rabbit anti-mouse IgG antibody labeled with horseradish peroxidase (DAKO Japan Co., Kyoto, Japan).

Deparaffinized sections were used in immunohistochemical analysis using a routine immunoperoxidase technique (indirect method). Sections were then rehydrated and blocked with 0.45% H_2O_2 in methanol for 30 minutes, followed by incubation with normal rabbit serum (DAKO Japan Co.) diluted to 1/50, for 20 minutes. The primary antibody, diluted to 1/50, was applied and incubated at 4°C overnight. After the sections were washed in PBS three times, the secondary antibody, diluted in 1/100 was applied sequentially for 30 minutes, followed by 0.2 mg/ml 3-3′ diaminobenzidine tetrahydrochloride (SIGMA-ALDRICH Co., Tokyo, Japan) in 50 mM Tris, pH 7.6, containing 0.01% H_2O_2 for 5 minutes. The sections were counterstained lightly with hematoxylin. The primary antibody was replaced by PBS for control sections.

Protein (MMP-1) Determination

We examined culture media taken from three dishes at 1, 3, 5, and 7 days. One series was from conditioned medium of cultured fibroblasts alone, another series was from cultured fibroblasts with MKN-74 conditioned medium.

Total MMP-1 protein in each culture media was measured by ELISA kit (Amersham International plc, Buckinghamshire, England). This assay is based on a two site ELISA sandwich format and specific for human total MMP-1. We showed the production of MMP-1 protein as optimal density (OD) per 10^4 cells at each plate.

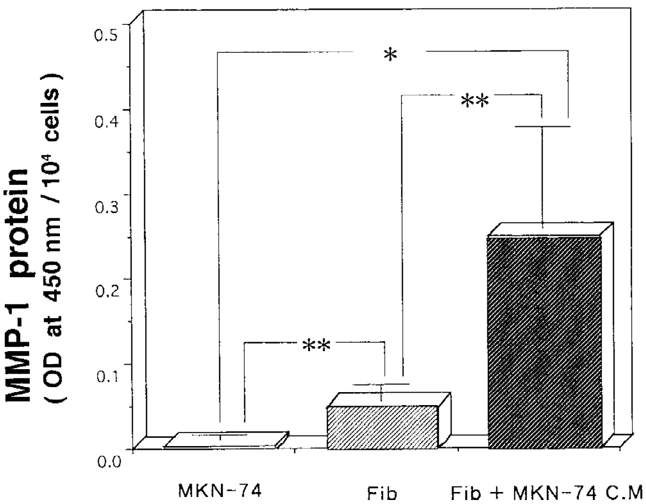

Fig. 1. Production of MMP-1 protein was not detected in the conditioned medium of MKN-74, while MMP-1 protein was detected to be 0.04 ± 0.01 in the conditioned medium of gastric fibroblasts alone. Production of MMP-1 protein in gastric fibroblasts was stimulated by the addition of the conditioned medium of MKN-74 (*P < 0.01; **P < 0.05).

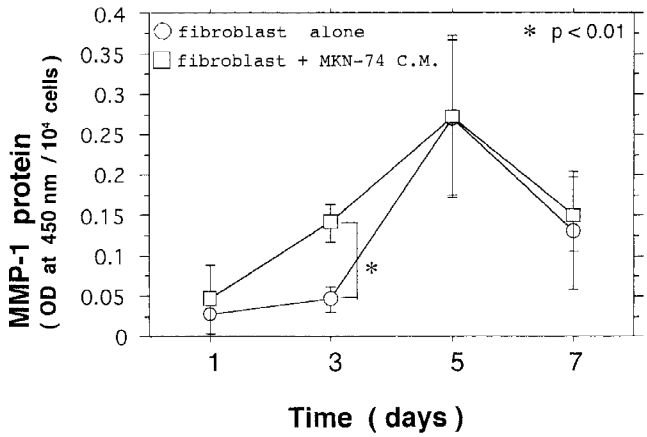

Fig. 2. Production of MMP-1 increased to the maximum level on 5 days after harvest, and on 3 days after harvest the degree of MMP-1 production was significantly higher in gastric fibroblasts with the conditioned medium of MKN-74 comparing with gastric fibroblasts alone (*P < 0.01).

Statistical Analysis

Statistical analysis was carried out by two group *t*-test (unpaired). If the *P*-value was smaller than 0.05, the difference was considered to be statistically significant.

RESULTS

MMP-1 Production

No MMP-1 was detected in the conditioned medium of MKN-74, while the level of MMP-1 protein was assessed to be 0.04 ± 0.01 in the conditioned medium of gastric fibroblasts alone. Production of MMP-1 protein in gastric fibroblasts was stimulated by adding the conditioned medium of MKN-74 with a statistically significant difference at $P < 0.05$ (Fig. 1). The production of MMP-1 increased to maximum level of 5 days after the harvest, and the increment of MMP-1 production was significantly higher in gastric fibroblasts with the conditioned medium of MKN-74 compared to gastric fibroblasts alone ($P < 0.01$, Fig. 2).

Localization of MMP-1 Protein in the Xenografted Gastric Cancer Cell Lines

In St-40, a well differentiated adenocarcinoma line, MMP-1 protein was not detected in the tumor cells, while MMP-1 was positive in the stroma of murine subcutaneous tissue (Fig. 3a). In St-4, a poorly differentiated adenocarcinoma line, MMP-1 protein was detected both in the tumor cells and stromal cells (Fig. 3b). MMP-1 positive cells were not detected in control sections. In this manner, the expression of MMP-1 protein was heterogenoeus according to different cell lines.

DISCUSSION

Interstitial collagenase (MMP-1), which degrades the triple helical domains of the fibrillar collagens (types I, II, III and X [7]), has been investigated in many human tumors. In the present study, fibroblasts derived from human gastric tissue produced MMP-1 protein, the production of which was stimulated by the addition of the conditioned medium from a gastric cancer cell line, MKN-74, although the MMP-1 protein was not detected in the conditioned medium of MKN-74 alone. These results suggested that the production of MMP-1 protein is regulated by interaction between stromal and tumor cells. The production of MMP-1 from other sources has been reported, including liver cells [8], synovial fibroblasts [9], and skin fibroblasts [10]. Since we have used orthotopic fibroblasts derived from human gastric tissue combined with gastric cancer cell line, the production of MMP-1 may differ from those using other fibroblasts originating from synovial or skin tissues. Growth regulation of tumor by the interaction between cancer cells and fibroblasts has been reported elsewhere [11–13]. It was reported that the co-inoculation of scirrhous gastric cancer cells with gastric fibroblasts into nude mice increased tumorigenicity, compared with that of gastric cancer cells alone, and conditioned medium from gastric fibroblasts stimulated the growth of cancer cells [14].

Studies on the regulative mechanism of MMPs expression suggest that several cytokines may control their transcription. The activity of MMPs in tumor-related stroma may be associated with IL-1 [15], TNF-α [16], IFN-γ [17], and TGF-β [18]. In our study, using human gastric cancer tissues, immunostaining for IL-1 was observed in granulocytes infiltrating into the stroma of neoplastic glands (data not shown).

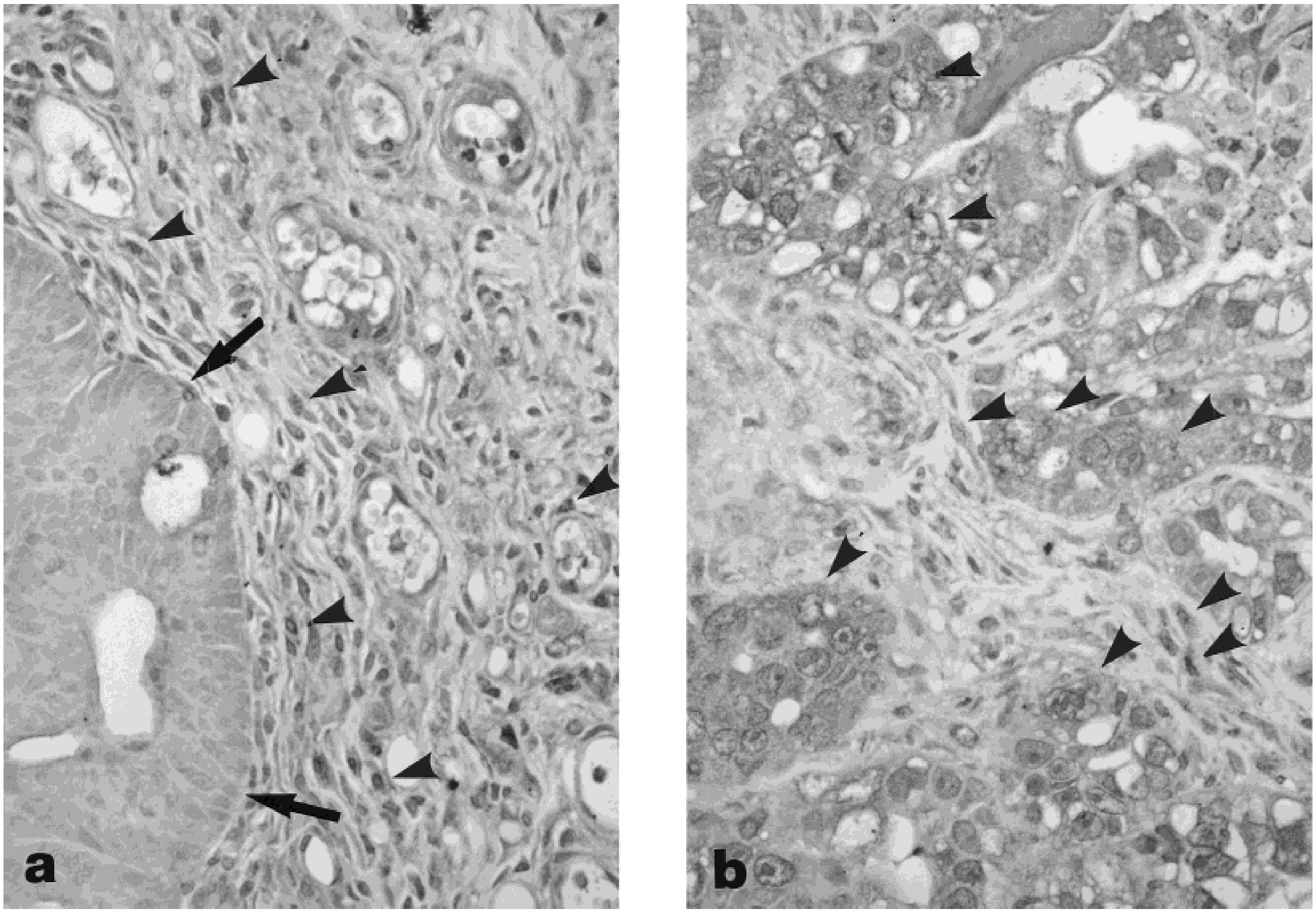

Fig. 3. Immunohistochemistry for MMP-1 protein. **a:** St-40, a well differentiated adenocarcinoma line. MMP-1 protein was not detected in the tumor cells (arrows), while MMP-1 positive cells were detected in the stromal cells (arrowheads). ×200, original magnification. **b:** St-4, a poorly differentiated adenocarcinoma line. MMP-1 protein was detected both in the tumor and stromal cells. Arrowheads indicate MMP-1 positive cells. ×200, original magnification.

The localization of MMP-1 positive cells in gastric cancer xenografts was heterogeneous according to different cell lines. MMP-1 protein was detected in St-4 tumor cells, while this enzyme was not detected in St-40 tumor cells. On the other hand, in the stroma of murine interstitial tissue, MMP-1 positive cells were observed in both strains. These different expressions of MMP-1 were comparable with our previous finding that the strong expression of MMP-1 mRNA has been observed in regions of moderately differentiated adenocarcinoma in human gastric cancer, whereas no expression was seen in the regions of poorly differentiated adenocarcinoma [19]. These phenomena suggested that the expression of MMP-1 protein is similar in the surgical specimens and in human tumor xenografts in nude mice. In this manner, the localization of MMP-1 may differ in histological and macroscopic types of gastric cancer, and MMP-1 may play a role in cancer invasion and tissue remodelling. To clarify the regulative mechanisms of MMP-1 expression in cancer tissue, further study is required.

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